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INTERACTIONS BETWEEN HEMLOCK WOOLLY
ADELGID, EASTERN HEMLOCK, AND GYPSY MOTH

BY

IAN GERARD KINAHAN

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF

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MASTER OF SCIENCE THESIS

OF

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UNIVERSITY OF RHODE ISLAND

2020

ABSTRACT

Eastern hemlock (*Tsuga canadensis*), a canopy-dominant conifer native to the eastern U.S., is currently threatened with extirpation by the invasive stylet-feeding hemlock woolly adelgid (*Adelges tsugae*). To understand interactions between eastern hemlock and hemlock woolly adelgid, and their interactions with other forest defoliators, we carried out two experiments.

First, in 2018, we evaluated the impact of feeding by hemlock woolly adelgid on gypsy moth (*Lymantria dispar*) larval preference for, and performance on, eastern hemlock. To assess preference, we surveyed 245 field-grown hemlocks for gypsy moth herbivory damage and conducted laboratory paired-choice bioassays. To assess performance, gypsy moth larvae were reared to pupation on adelgid-infested or uninfested hemlock foliage and pupal weight, proportional weight gain, and larval period were analyzed. Adelgid-infested hemlocks experienced more gypsy moth herbivory than uninfested control trees, and laboratory tests confirmed that gypsy moth larvae preferentially feed on adelgid-infested hemlock foliage. Gypsy moth larvae reared to pupation on adelgid-infested foliage gained more weight than larvae reared on uninfested control foliage. Our results suggest that the synergistic effect of adelgid and gypsy moth poses an additional threat to eastern hemlock that may increase extirpation risk and ecological impact throughout most of its range.

Second, we have conducted over a decade of research into rare eastern hemlock (*Tsuga canadensis*; hemlock) trees that appear resistant to hemlock woolly adelgid (*Adelges tsugae*; HWA). Following clonal propagation of these rare individuals, in 2015 we planted size- and age-matched HWA-resistant and HWA-susceptible

hemlocks in HWA-infested forest plots in seven states. In 2019, we re-surveyed the plots; 96% of HWA-resistant hemlocks survived compared to 48% of susceptible trees. The surviving HWA-resistant trees were also taller, produced more lateral growth, retained more foliage, and supported lower elongate hemlock scale (*Fiorinia externa*) and HWA densities than the surviving HWA-susceptible trees. Our results suggest that HWA management may benefit from additional research exploring the identification, characterization, and use of HWA-resistant eastern hemlocks in future reforestation efforts.

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PREFACE

The following thesis will be submitted as two chapters, in manuscript format following the formatting guidelines of the scientific journals: 1. Ecological Entomology, and 2. Forests.

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In press Ecological Entomology, 2020

**Facilitation between invasive herbivores: hemlock woolly adelgid increases
gypsy moth preference for and performance on eastern hemlock**

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Abstract.

1. Interactions between invertebrate herbivores with different feeding modes are common on long-lived woody plants. In cases where one herbivore facilitates the success of another, the consequences for their shared host plant may be severe. Eastern hemlock (*Tsuga canadensis*), a canopy-dominant conifer native to the eastern U.S., is currently threatened with extirpation by the invasive stylet-feeding hemlock woolly adelgid (*Adelges tsugae*). The effect of adelgid on invasive hemlock-feeding folivores remains unknown.

2. We evaluated the impact of feeding by hemlock woolly adelgid on gypsy moth (*Lymantria dispar*) larval preference for, and performance on, eastern hemlock. To assess preference, we surveyed 245 field-grown hemlocks for gypsy moth herbivory damage and conducted laboratory paired-choice bioassays. To assess performance, gypsy moth larvae were reared to pupation on adelgid-infested or uninfested hemlock foliage and pupal weight, proportional weight gain, and larval period were analyzed.

3. Adelgid-infested hemlocks experienced more gypsy moth herbivory than uninfested control trees, and laboratory tests confirmed that gypsy moth larvae preferentially feed on adelgid-infested hemlock foliage. Gypsy moth larvae reared to pupation on adelgid-infested foliage gained more weight than larvae reared on uninfested control foliage.

4. Our results suggest that the synergistic effect of adelgid and gypsy moth poses an additional threat to eastern hemlock that may increase extirpation risk and ecological impact throughout most of its range.

Key words. Facilitation, herbivores, *Adelges tsugae*, *Lymantria dispar*, invasional meltdown

Introduction

Many interactions between co-occurring insect herbivores are mediated by their impact on the shared host plant (Kaplan & Denno, 2007). Feeding by one insect may cause alterations in plant quality, such as the induction of toxic secondary metabolites or changes to various leaf structural traits, which can affect simultaneously- or sequentially-feeding competitors (Nykänen & Koricheva, 2004). Although many such changes negatively impact the other species, they can also be facilitative (Kaplan & Denno, 2007; Ohgushi, 2008). Sap feeding by the aphid *Brevicoryne brassicae*, for example, improves the performance of folivorous *Pieris brassicae* larvae by attenuating chemical defense induction in *Brassica oleracea* (Li *et al.*, 2014).

Understanding herbivore-herbivore interactions is especially important in cases where one or both herbivores can substantially affect plant growth and fitness. One such species is hemlock woolly adelgid (*Adelges tsugae*; ‘adelgid’ hereafter), a destructive pest that has caused widespread mortality and decline of an ecologically significant conifer, eastern hemlock (*Tsuga canadensis*; ‘hemlock’ hereafter), in eastern U.S. forests. Adelgid feeds by inserting its stylet bundle into the xylem ray parenchyma cells at the base of a hemlock needle (Shields *et al.*, 1995). This feeding reduces the production of new foliage (Gonda-King *et al.*, 2014; McClure, 1991), alters wood morphology (Domec *et al.*, 2013; Gonda-King *et al.*, 2012), and substantially impacts plant physiology. Adelgid-infested hemlocks have elevated tissue levels of salicylic acid (SA) and emissions of its methylated form, methyl salicylate (Pezet *et al.*, 2013; Pezet & Elkinton, 2014). SA is a phytohormone that

plays a critical role in plant response to abiotic stresses and biotrophic pathogens; it has also been shown to accumulate following stylet-feeding insect infestations (Walling, 2000). SA accumulation and subsequent monomerization of NPR1, a transcriptional regulator that promotes the expression of SA-responsive genes, can interfere with the biosynthesis of jasmonic acid (JA)/ethylene-dependent defenses that help protect against leaf-chewing herbivores (Walling, 2008; Zarate *et al.*, 2007). Adelgid feeding has also been shown to increase nitrogen (Gonda-King *et al.*, 2014) and total amino acid content (Gomez *et al.*, 2012) in hemlock needles. Because nitrogen is critical to insect growth (Awmack & Leather, 2002; Kerslake *et al.*, 1998), such adelgid-mediated increases may enhance host plant quality for folivorous herbivores.

Recent work in the hemlock system suggests that adelgid-induced phytochemical changes may influence interactions between hemlock and other herbivores (Rigsby *et al.*, 2019; Schaeffer *et al.*, 2018; Wilson *et al.*, 2018). Larvae of the native hemlock looper (*Lambdina fiscellaria*) had higher survival and enhanced larval development when reared on adelgid-infested versus uninfested hemlock foliage (Wilson *et al.*, 2016). This work led us to explore whether similar interactions might be occurring between the adelgid and more commonly-occurring folivores. We focused our attention on gypsy moth (*Lymantria dispar*), an invasive folivore that has devastated eastern U.S. forests. Since its introduction in 1890, periodic gypsy moth outbreaks have defoliated millions of acres and altered forest structure and composition (Gandhi & Herms, 2010; Lovett *et al.*, 2006). Gypsy moth can feed on

eastern hemlock (Lovett *et al.*, 2006) and although it and hemlock woolly adelgid co-occur in their introduced range, their interactions have not been considered.

We report the results of work assessing the impact of adelgid infestation on gypsy moth-hemlock interactions. We surveyed hemlocks planted into a deciduous forest understory for gypsy moth herbivory and conducted two laboratory experiments to measure gypsy moth preference for, and performance on, adelgid-infested hemlock foliage. Because the adelgid inhibits hemlock anti-folivore defense pathways and increases the nutritional value of its needles, we hypothesized that gypsy moth larvae would both prefer (consume more of) and do better (pupate at higher weights) on adelgid-infested foliage. The ‘invasional meltdown hypothesis’ suggests that much of the damage caused by introduced species may result from positive interactions between invaders that can facilitate their establishment and increase their ecological impact (Simberloff & Von Holle, 1999). Our findings illustrate the potential for such facilitation between two invasive herbivores and highlight the threat this may pose to their shared host and its associated ecosystem.

Materials and Methods

Field preference survey: Our field preference survey took advantage of a 2016 gypsy moth outbreak to assess their impacts on field-grown eastern hemlock. The trees in this survey were planted in 2014 for use in an unrelated experiment. Briefly, 1-1.2 m-tall hemlock saplings were purchased from Van Pines Nursery (West Olive, MI) in spring 2014, planted, and grown for two years in the understory of a mixed hardwood stand at the Kingston Wildlife Research Station (South Kingstown, RI). Hemlocks were planted in five 64-tree blocks, with each tree spaced 1-1.5 m apart. Trees were

protected from herbivory and cross-contamination of treatments with chicken-wire cages covered by mesh bags (Agribon-15, Johnny's Selected Seeds, Waterville, ME, USA; 90% light transmission). Sixteen trees in each block were randomly assigned one of the following two treatments: infestation with adelgid or another invasive herbivore (*Fiorinia externa*; elongate hemlock scale, 'scale' hereafter). The remaining 32 trees in each block were maintained as controls. Trees in the adelgid and scale infestation treatments were inoculated in the spring of 2014, 2015, 2016, and 2017 with infested foliage collected from nearby adelgid-infested and scale-infested hemlocks, respectively; trees in the control treatment had herbivore-free hemlock foliage placed on them to control for disturbance.

In spring 2016, a gypsy moth outbreak occurred at our field site. Fourth-instar gypsy moth larvae were regularly seen roaming on the ground, where they could crawl under the mesh bags enclosing our trees. Over a short (2-3 week) time period, we observed that many of our trees received substantial damage from gypsy moth larvae. In late June 2016, 69 trees in the adelgid-infested treatment group, 69 trees in the scale-infested treatment group, and 107 trees in the control treatment group were assessed for gypsy moth herbivory damage, for a total of 245 trees. All branches emerging from the main stem of each tree were surveyed, and each tree was given a combined damage score of 0-3 (0=0-25% foliage loss, 1=26-50%, 2=51-75%, 3=76-100%). An annual, early spring survey confirmed that trees did not experience foliage loss prior to the gypsy moth outbreak. During the survey, fourth- and fifth-instar gypsy moth larvae were confirmed to be the only folivores present on trees.

Laboratory preference assay: Hemlock foliage used in the laboratory preference assay came from 0.5-0.7m hemlock saplings purchased from Vans Pines Nursery (West Olive, MI) in spring 2016. In late spring 2016, we inoculated half of the trees with adelgid-infested foliage from nearby trees; we attached adelgid-free hemlock foliage to the other trees (the control group) to control for disturbance. All trees were covered in mesh (Agribon-15, Johnny's Selected Seeds, Waterville, ME, USA; 90% light transmission) to prevent cross-contamination between treatments and grown in 1-gallon pots outside of the greenhouse complex at the University of Rhode Island (URI; Kingston, RI). Adelgid densities on each tree were assessed in late fall 2016 and early spring 2017. Five secondary branches on each tree were randomly selected, and all adelgid present on the branches were counted. We used this data to ensure that both the trees and specific branches used in this experiment had similar adelgid densities (0.8-1 adelgid/cm).

In late spring 2017, we collected 40 gypsy moth larvae from a mixed-hardwood forest located adjacent to the URI greenhouses. Having observed mostly 4th-5th instar larvae on our hemlocks in the field survey, we collected 4th-5th instar larvae found wandering on the ground or on tree trunks; all larvae were similarly-sized and highly active throughout the experiment. To assess gypsy moth preference for adelgid-infested hemlock, we collected 40 ~10cm terminal branches: one branch from each of 20 adelgid-infested trees, and one branch from each of 20 uninfested trees. Each branch was weighed; analysis *via* two-tailed Welch's unequal variances t-test confirmed there was no significant difference in the mean branch weight experienced by larvae in each treatment group ($t_{36} = -0.72$, $P = 0.4731$). Following weighing, the

branches were inserted into individual blocks of water-saturated floral foam (Oasis brand, Kent, OH). Two pieces of foliage (one adelgid-infested and one control) were then put in a 6L polypropylene bin (Sterilite brand, Townsend, MA). The pieces of foliage were placed at the 25% and 75% marks between the left and right sides of the bin; treatment placement was alternated between left and right. After two similarly-sized gypsy moth larvae were weighed, they were both added to the center of each bin. Each bin was then covered with metal mesh held in place by a rubber band. There were a total of 20 bins in the experiment. After one day, the mesh was removed and the larvae and foliage (including any dropped needles) were weighed; the adelgid-infested and uninfested foliage were weighed separately to calculate larval consumption for each treatment.

Laboratory performance assay: Hemlock foliage used in the laboratory performance assay came from the common garden planting described in the field preference survey. In late spring 2018, 3rd-instar gypsy moth larvae were obtained from the USDA-APHIS Laboratory in Buzzards Bay, MA. These larvae originated from the New Jersey Standard Strain-APHIS substrain, a laboratory colony which has been in cultivation for >60 generations. Larvae were reared on an artificial diet (Frontier Agricultural Sciences, USDA Hamden Formula) until they reached the fourth instar, at which point each larva was weighed and placed individually into one of 50 473 ml glass mason jars (Ball brand, Broomfield CO). Fourth-instar larvae were used in this experiment because younger stages have trouble consuming hemlock foliage, likely because their undeveloped mouthparts cannot penetrate lignified needles. By contrast, larvae in the fourth instar and above readily consume hemlock.

Half of the jars contained foliage from adelgid-infested hemlocks, while the other half of the jars contained foliage from uninfested hemlocks, for a total of 25 replicates per treatment. The foliage in each jar consisted of a single ~17cm sprig of foliage kept upright in hydrated floral foam (Oasis brand, Kent, OH); foliage was checked every day and replaced if >50% of the needles had been consumed. The top of each jar was covered with nylon mesh and all jars were kept in a growth chamber (15:9 L:D, 24°C, 60-70% RH). Larvae were checked every two days and the position of the jars rotated within the growth chamber; the date of and weight at pupation was recorded for each individual.

Statistical analysis: All data were inspected for normality (Shapiro-Wilk test) and homoscedasticity (Bartlett's test) prior to analysis; data were log-transformed where necessary to meet assumptions. Damage scores were tabulated by treatment group and analyzed *via* Pearson's chi-squared test. Data from the laboratory preference assay were analyzed *via* two-tailed Welch's unequal variances t-test. Percent weight gain, pupal weight and larval period were analyzed separately *via* three-way analysis of covariance (ANCOVA), with foliage type and sex as the predictors, initial larval weight as a covariate, and all two-way interactions. We classified larvae as male or female because the sexes differ substantially in their time to and weight at pupation (Myers et al., 1998); this allowed us to analyze percent weight gain, pupal weight, and larval period of the two sexes separately for both foliage treatment groups. Tukey's test was used to separate the mean response of the two sexes in either foliage treatment group. Figures were created using *ggplot2*

(Wickham, 2016). R software v. 3.5.0 was used for all statistical analyses (R Development Core Team, 2018).

Results

Field preference survey: Adelgid-infested hemlocks experienced significantly more gypsy moth herbivory damage than scale-infested or control trees ($X^2 = 48.96$, $P < 0.0001$; Fig. 1). Nearly 40% of adelgid-infested trees lost more than half of their foliage to gypsy moth herbivory, while fewer than 10% of scale-infested trees and 5% of control trees experienced similar levels of damage. Conversely, 84% of both control and scale-infested trees experienced minimal (0-25% foliage loss) herbivory.

Laboratory preference assay: When allowed to choose between adelgid-infested and control foliage, larvae consumed an average of 37% more adelgid-infested foliage than control foliage (0.36 g \pm 0.054 SE and 0.22 g \pm 0.034 SE, respectively; $t_{31} = -2.17$, $P = 0.0380$).

Laboratory performance assay: Larvae reared to pupation on adelgid-infested hemlock foliage gained more weight, and pupated at a higher weight, than larvae reared on uninfested foliage (both $P < 0.05$; Fig. 2 A, B). Female larvae gained more weight, pupated at a higher weight, and took longer to pupate than male larvae (all $P < 0.05$; Fig. 2 A, B, C). Initial larval weight affected larval weight gain and weight at pupation, but not larval period.

Female larvae reared on adelgid-infested foliage gained 256% of their initial weight, while those fed control foliage gained 120% of their initial weight ($P < 0.001$; Fig. 2 A). Male larvae reared on adelgid-infested and uninfested foliage gained 115% and 67% of their initial weight, respectively ($P < 0.001$; Fig. 2 A).

Female larvae reared on adelgid-infested foliage pupated at weights 25% greater than those reared on uninfested foliage ($F_{1,36} = 12.5$, $P = 0.0011$; Fig. 2 B). Conversely, male larvae reared on adelgid-infested and control foliage pupated at similar weights ($P = 0.88$; Fig. 2 B).

Larval period was not affected by treatment or initial weight (both $P > 0.4$; Fig. 2 C), although female larvae reared on adelgid-infested hemlock foliage had a larval period five days longer than that of male larvae reared on uninfested foliage ($P = 0.0249$; Fig. 2 C).

Discussion

Here we present evidence that one destructive forest pest, hemlock woolly adelgid, facilitates the development of the invasive gypsy moth. We found that gypsy moth larvae prefer hemlock foliage infested with hemlock woolly adelgid (Fig. 1), and that feeding on this infested foliage facilitates gypsy moth larval development. Female larvae reared on adelgid-infested hemlock foliage gained more than twice as much of their initial weight (Fig. 2 A) and pupated at 25% higher weights (Fig. 2 B) than larvae reared on uninfested foliage. Male larvae reared to pupation on adelgid-infested foliage also gained 48% more weight than those fed uninfested foliage (Fig. 2 A) but pupated similar weights (Fig. 2 B). Additionally, gypsy moth larvae exhibited a preference for adelgid-infested foliage over uninfested foliage, and in a natural setting, adelgid-infested hemlocks experienced substantially more gypsy moth herbivory than uninfested trees. Our results are consistent with findings from a previous study (Wilson *et al.* 2016) documenting a facilitative effect of hemlock woolly adelgid on another leaf-chewing herbivore.

The enhanced performance of gypsy moth larvae reared on adelgid-infested foliage may result from adelgid-induced changes to hemlock defenses. Adelgid infestation of hemlock increases foliar emissions of methyl salicylic acid (Pezet *et al.*, 2013; Pezet & Elkinton, 2014) and triggers salicylic acid (SA) accumulation in needles (Schaeffer *et al.*, 2018; Rigsby *et al.*, 2019), activating SA-linked stress responses in hemlock. The accumulation of SA, and subsequent monomerization of NPR1, has been shown to inhibit jasmonic acid (JA) biosynthesis and JA-responsive gene expression (Zarate *et al.*, 2007). Plant defense against leaf-chewing herbivores is primarily mediated by JA (Gilbert & Liebhold, 2010; Kroes *et al.*, 2014), and blocking the induction of JA-related defenses may make the foliage of adelgid-infested hemlocks more digestible and/or easily converted into body mass by gypsy moth larvae. This interpretation is supported by work on other systems where negative cross-talk between these pathways has been shown to improve the performance of a later-arriving herbivore (reviewed in Stam *et al.*, 2014).

The improved performance of gypsy moth may also be driven by enhanced foliar nutritional quality in adelgid-infested hemlocks. Hemlock needles on adelgid-infested stems are higher in nitrogen, suggesting that hemlock woolly adelgid may turn needles into nitrogen-rich sinks. For instance, amino acid content in adelgid-infested hemlock foliage has been measured at levels 3.3-fold greater than uninfested foliage (Gomez *et al.*, 2012). Nitrogen plays a key role in the development and fecundity of herbivorous insects (Awmack & Leather, 2002; Kerslake *et al.*, 1998). High concentrations of dietary nitrogen have been shown to increase gypsy moth larval survival and pupal weights (Lindroth *et al.*, 1997), and gypsy moth fecundity

has been positively correlated with host plant foliar nitrogen content (Hough & Pimentel, 1978). This is consistent with prior work in this system by Wilson *et al.* (2016) that found hemlock looper larvae reared on adelgid-infested foliage had higher early-instar survival and attained higher pupal weights than larvae reared on uninfested foliage.

Adelgid-infested hemlocks in our common garden planting experienced significantly higher rates of defoliation compared to both control (herbivore-free) and scale-infested trees (Fig. 1). Laboratory choice-assays confirmed that gypsy moth larvae preferentially feed on adelgid-infested hemlock foliage. In addition to documenting increased overall nitrogen and amino acid concentrations in adelgid-infested hemlocks, Gomez *et al.* (2012) reported substantial increases in levels of the amino acid proline. Proline can act as an indicator of plant stress (Mattson & Haack, 1987), and is an important source of stored energy for insects (Gäde & Auerswald, 2002). In this case, elevated proline content in adelgid-infested hemlocks may act as a phagostimulatory signal of vulnerability and elevated nutrient content. This pattern has been documented in other plant-insect systems, particularly for various Hemiptera, Lepidoptera, Orthoptera and mite species (Mattson & Haack, 1987).

The fact that adelgid feeding enhances gypsy moth preference for, and performance on, eastern hemlock, makes it likely that their co-occurrence on hemlock can additively stress and further threaten this important conifer. In southern New England, adelgid infestation has caused extensive mortality of overstory hemlocks (Eschtruth *et al.*, 2006; Orwig *et al.*, 2002; Preisser *et al.*, 2008), altering understory conditions that put hemlock seedlings at a competitive disadvantage (Orwig *et al.*,

2013; Orwig *et al.*, 2008). Hemlocks are adapted to cool microclimates and low light levels (Hadley, 2000), and increased light exposure due to crown thinning and mortality of mature trees inhibits recruitment of hemlock seedlings and favors establishment of black birch (*Betula lenta*) and other deciduous trees (Ingwell *et al.*, 2012; Orwig & Foster, 1998; Orwig *et al.*, 2002). Preferential feeding by gypsy moth larvae on adelgid-infested overstory hemlocks may exacerbate this effect, reducing the likelihood of new hemlock recruits eventually repopulating devastated hemlock forests. The damage inflicted by gypsy moths on adelgid-infested hemlock saplings may further compromise regeneration. Over a four-year period, hemlock regeneration in adelgid-infested forests declined by 46% (Preisser *et al.*, 2011). Feeding by both species may accelerate this decline, if inhibited seedling recruitment is coupled with significant mortality of juvenile hemlock saplings.

Enhanced performance of gypsy moth larvae on adelgid-infested hemlock may also have a cascading effect on other plant taxa that grow with hemlock in forests of the eastern U.S. Oaks (*Quercus* spp.) are a preferred host of gypsy moth (Barbosa *et al.*, 1979; Hough & Pimentel, 1978), and feeding by gypsy moth larvae has caused extensive mortality and decline of overstory oaks throughout this region (Gandhi & Herms, 2010). Total basal area of overstory oaks has decreased due to gypsy moth herbivory, and mortality of white oak (*Quercus alba*), northern red oak (*Quercus rubra*), and chestnut oak (*Quercus montana*) specifically has increased by 40% (Fajvan & Wood, 1976). Gypsy moth herbivory in southern New England forests has increased oak mortality and reduced the growth of surviving canopy trees by as much as 65% (Gottschalk *et al.*, 1990). It is plausible that the enhanced growth of female

gypsy moth larvae on adelgid-infested hemlock may translate to greater fecundity, which could increase gypsy moth population densities in southern New England forests. Since tree mortality increases as the intensity and frequency of gypsy moth defoliation increases (Davidson *et al.*, 1999), larger gypsy moth populations here could speed oak decline.

It is important to realize that ecological traits of the gypsy moth larvae used in the laboratory performance assay may not be comparable with those of wild gypsy moth larvae. Larvae used in the laboratory performance assay were part of the New Jersey Standard Strain-APHIS substrain, a mass-reared colony of gypsy moth larvae that has been in cultivation for >60 generations. Because this colony is intended for research, certain selective regimes and control measures have been enacted upon it to maximize the survival and fecundity of the gypsy moths. These include laboratory selection for higher survival and fecundity, and an artificial diet (Frontier Agricultural Sciences, USDA Hamden Formula), which may incidentally select for genotypes that show reduced performance on a natural diet (Grayson *et al.*, 2015). However, a comparison of development between gypsy moth larvae from the New Jersey Standard Strain-APHIS substrain, -FS substrain, and three wild populations all reared on a natural diet, found no population-level differences in male and female pupal weights (Grayson *et al.*, 2015). Additionally, our observation of substantial wild gypsy moth larval herbivory damage to adelgid-infested field-grown eastern hemlocks, as well as a confirmed wild gypsy moth larval preference for adelgid-infested hemlocks, mirror results from the laboratory performance assay and further support their ecological relevance.

Adelgid-induced hemlock mortality has severely affected ecosystem dynamics in eastern U.S. forests. Hemlock supports critical habitat for unique vertebrate and invertebrate communities (Ellison *et al.*, 2010), and dramatic shifts in understory vegetation, soil nutrient cycling and hydrological regimes may have long-lasting changes that compromise these areas (Orwig *et al.*, 2008). Future work should evaluate the extent to which adelgid and gypsy moth act synergistically to speed the decline of eastern hemlock and other canopy-dominant species, and the impact this could have on hemlock-associated ecosystems.

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There are no disputes over the ownership of the data presented in this paper. All contributions have been attributed appropriately, via coauthorship or acknowledgement, as appropriate to the situation.

Contribution of Authors

IGK, ELP, CMR, and CMO were responsible for project design. IGK, AKB, ERW, SKS, EES, and ELP were responsible for data collection and analysis. IGK, ELP, AKB, CMR, and CMO were responsible for paper writing.

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Figure Legends

Figure 1. Gypsy moth larval herbivory damage to eastern hemlocks in adelgid (*Adelges tsugae*)-infested, uninfested control, and scale (*Fiorinia externa*)-infested treatment groups. Damage was quantified on a scale of 0-3, representing % foliage loss of trees in each treatment group (0=0-25% foliage loss, 1=26-50%, 2=51-75%, 3=76-100%).

Figure 2. Percent weight gain (A), pupal weight (B), and larval period (C) of gypsy moth larvae reared on either adelgid-infested or uninfested control hemlock foliage. Bars represent means \pm 1 SE; F = female larvae, M = male larvae. Capital letters denote significant treatment-level differences ($P < 0.05$).

Figure 1.

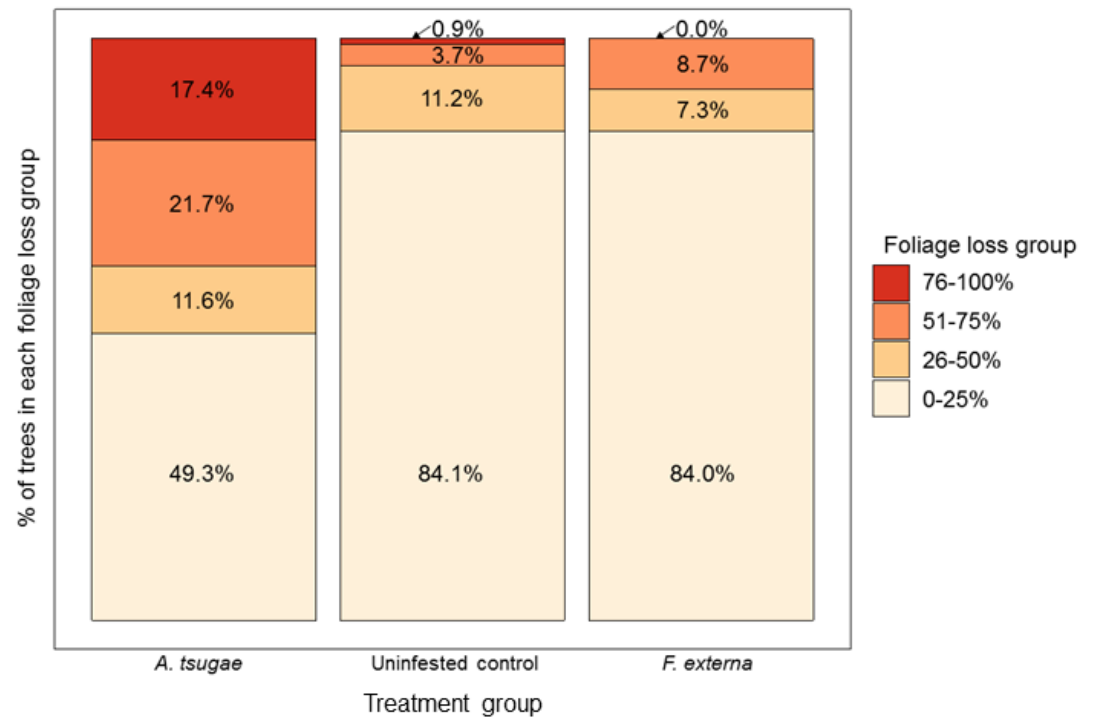
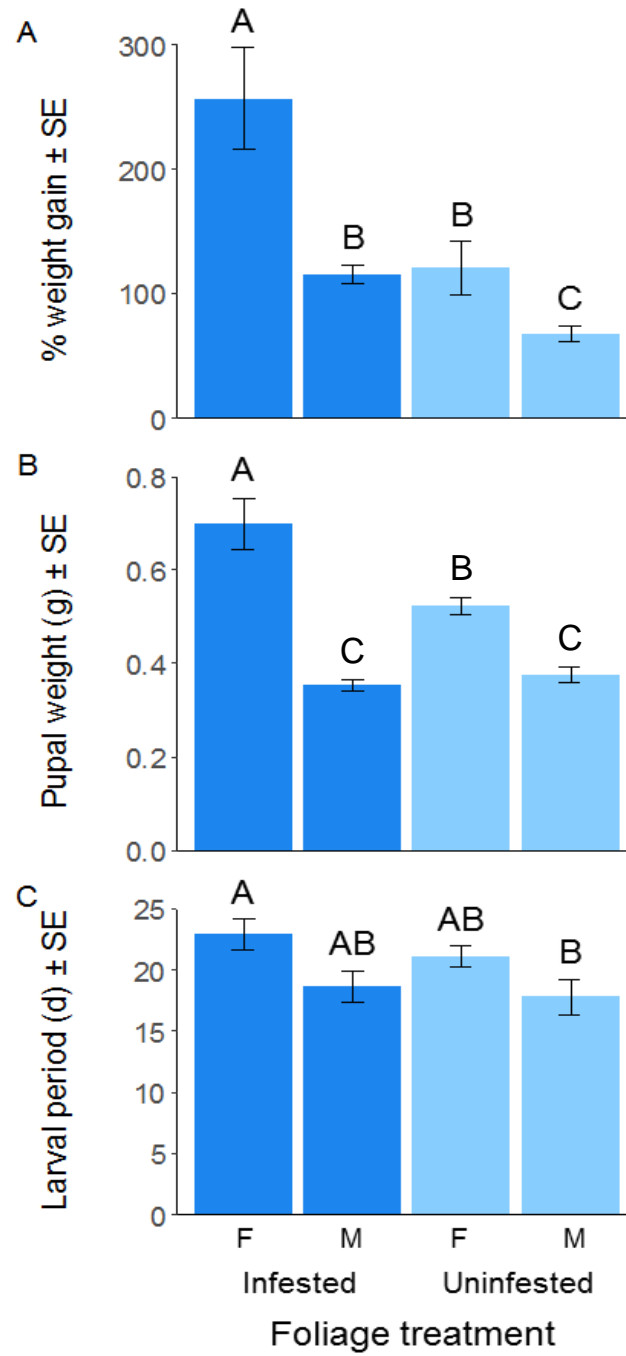


Figure 2.



MANUSCRIPT - 2

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A four-year, seven-state reforestation trial with eastern hemlocks (*Tsuga canadensis*)
resistant to hemlock woolly adelgid (*Adelges tsugae*)

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Abstract

We have conducted over a decade of research into rare eastern hemlock (*Tsuga canadensis*; hemlock) trees that appear resistant to hemlock woolly adelgid (*Adelges tsugae*; HWA). Following clonal propagation of these rare individuals, in 2015 we planted size- and age-matched HWA-resistant and HWA-susceptible hemlocks in HWA-infested forest plots in seven states. In 2019, we re-surveyed the plots; 96% of HWA-resistant hemlocks survived compared to 48% of susceptible trees. The surviving HWA-resistant trees were also taller, produced more lateral growth, retained more foliage, and supported lower pest densities than the surviving HWA-susceptible trees. Our results suggest that HWA management may benefit from additional research exploring the identification, characterization, and use of HWA-resistant eastern hemlocks in future reforestation efforts.

Key words

Eastern hemlock, hemlock woolly adelgid, host, plant, resistance

Introduction

Although chemical suppression and biological control efforts are often the primary tools for managing non-native forest insects and pathogens (hereafter, ‘pests’), research that assesses and exploits the potential for pest resistance in host plant populations has played an integral role in many forest pest management programs (Sharma and Ortiz 2002). Dutch elm disease (*Ophiostoma ulmi* and *Ophiostoma novo-ulmi*; hereafter, ‘DED’), for instance, is the most destructive pest of shade-trees in the United States (Schlarbaum et al. 1998), having wiped out nearly 100 million American elms (*Ulmus americana*) in both urban and forest populations

(Karnosky 1979). Healthy, mature individual American elms persist, however, in DED-devastated forests throughout the eastern U.S. (Schlarbaum et al. 1998), and research suggests that these trees are less susceptible to the disease (Townsend et al. 2005). Production and evaluation of clonal propagules from these trees has yielded several DED-tolerant American elm genotypes that are commercially available (Townsend et al. 2005) and currently being used in ecosystem restoration (Knight et al. 2012). Similarly, chestnut blight (*Cryphonectria parasitica*) has rendered the American chestnut (*Castanea dentata*) functionally extinct throughout most of its range (Paillet 2002). While biological control has proven relatively unsuccessful in population-level management of this pest (reviewed in Milgroom and Cortesi 2004), research on rare, surviving individual American chestnuts has identified some degree of blight resistance in these trees (reviewed in Jacobs et al. 2013). Several ongoing breeding programs utilizing backcrossing of lingering American chestnuts with blight-resistant Chinese chestnuts (*C. mollissima*) have produced relatively blight-resistant individuals that are phenotypically indistinguishable from American chestnuts (Cipollini et al. 2017). Preliminary reforestation trials indicate that these blight-resistant American chestnut hybrids may well prove critical in restoring chestnut to blight-devastated forests of the eastern U.S. (reviewed in Knight et al. 2017).

Research examining host plant resistance is especially important in cases where a pest cannot be effectively controlled by biological control or insecticides (Hanover 1975; Oten et al. 2014; Showalter et al. 2018). One such pest is hemlock woolly adelgid (*Adelges tsugae*; hereafter, ‘HWA’), a hemipteran accidentally introduced from Japan approximately 70 years ago (Havill et al. 2006) that has caused

widespread mortality and decline of eastern hemlock (*Tsuga canadensis*; hemlock) throughout eastern U.S. forests. While insecticides can protect individual trees or small stands, they must be re-applied periodically and are impractical for forest-level application. Although several HWA biological control agents have been approved for release and others are currently being evaluated, evidence is mixed regarding their effect on forest health (Sumpter et al. 2018). At the same time, there is ample evidence of interspecific variation in hemlock resistance to HWA (Lagalante and Montgomery 2003; Lagalante et al. 2007). Chinese hemlock (*T. chinensis*) has repeatedly been shown to be resistant (Del Tredici and Kitajima 2004; Hoover et al. 2009; Montgomery et al. 2009), while western hemlock (*T. heterophylla*) and other Asian hemlock species sustain lower HWA densities (Montgomery et al. 2009) and possess foliar terpene profiles that differ substantially from those of HWA-susceptible hemlock species (Lagalante and Montgomery 2003). Researchers have attempted to take advantage of this variation by crossing both HWA-susceptible hemlock species (*T. canadensis* and *T. caroliniana*) with HWA-resistant hemlocks; unfortunately, none of the *T. canadensis* crosses produced viable offspring (Bentz et al. 2002).

The fact that other *Tsuga* species possess adelgid resistance suggests that this trait has at least the potential to occur in rare *T. canadensis*. This argument was supported by work showing that the foliar terpenes (chemicals used by the plant to defend against HWA and other pests) found in several dwarf *T. canadensis* cultivars differ substantially from those of ‘wild’ *T. canadensis* (Lagalante et al. 2007). This work inspired two of us (RC and EP) to explore the potential for variation in HWA resistance within *T. canadensis*. We created and distributed a pamphlet asking forest

managers and other concerned groups for their help in locating and identifying eastern hemlocks that appeared to be HWA-resistant (Ingwell and Preisser 2011). Qualifying trees had to be mature eastern hemlocks that appeared completely healthy, were located within HWA-devastated hemlock stands, and had not been chemically treated. This effort and additional discussions with HWA researchers led to the identification of a small stand of eastern hemlocks growing within the Walpack Fish and Wildlife Management Area in northern New Jersey, USA (Ingwell and Preisser 2011). Many trees within this forest (nicknamed the “bulletproof stand” by the NJ Department of Environmental Protection employee who found it) were mature and appeared healthy, with deep green foliage and little to no HWA infestation, despite growing in an HWA-devastated forest where HWA had been present for over 30 years. We evaluated HWA resistance in five of these trees via inoculation of rooted stem cuttings with HWA; after a three-month period, progrediens densities on HWA-resistant eastern hemlock propagules were significantly lower than those on HWA-susceptible propagules (Ingwell and Preisser 2011). Subsequent chemical analyses of foliage samples collected from parent HWA-resistant hemlocks *in situ* found significantly higher terpene concentrations in their twigs and needles than in the twigs and needles of HWA-susceptible hemlocks growing within a five-mile radius (McKenzie et al. 2014). While this result may have reflected different site conditions, they also reported on repeated analyses of foliage samples from two-year old clonal propagules from the same two tree populations. Although both HWA-resistant and HWA-susceptible plants were grown under identical conditions in an outdoor raised bed, they found that the HWA-resistant trees had higher concentrations of all 22 measured terpenes in their

twigs and needles. The authors suggest that these higher concentrations may provide one potential mechanism for these trees' reduced vulnerability to HWA (McKenzie et al. 2014).

We present the results of a four-year, seven-state reforestation trial with clonally-propagated HWA-resistant eastern hemlocks. Resistant and susceptible trees were planted in HWA-infested forest plots at varying latitudes; survival, growth, and pest infestation were evaluated. Four years after establishment, we found that HWA-resistant eastern hemlocks had higher survival, better growth characteristics, and lower pest densities than susceptible trees. Our results suggest that reforestation efforts with HWA-resistant eastern hemlocks may prove useful in HWA management efforts.

Methods

Source trees

All HWA-resistant propagules used in this study were collected from parent trees #3 and #4 growing in the “Bulletproof Stand” in the Walpack Fish and Wildlife Management Area in northern New Jersey, USA (Ingwell and Preisser 2011). For susceptible controls, we purchased saplings from areas not yet infested with HWA: Evergreen Nursery, Sturgeon Bay, WI (seed source: Upper Peninsula of Michigan, USA,) and Vans Pines Nursery, West Olive, MI (seed source Indiana County, PA, USA). This genetic source of hemlock has been shown to be susceptible in HWA in unrelated experiments (e.g. Wilson et al. 2018). In May 2011, both nurseries provided 20-30 cm saplings which were potted into 4 liter nursery containers with Metro Mix 830 growing media (Sungro Horticulture, Agawam, MA, USA) and grown outdoors between greenhouses under a 70% shade cloth for several years.

Greenhouse propagation

In January 2011, branch cuttings of 20 cm terminal growth were taken from parent HWA-resistant trees #3 and #4 at approximately 3 m height. Cuttings were placed on ice and immediately transported to the University of Rhode Island (Kingston, RI, USA), where they were stored in a walk-in cooler at 4.4° C for no more than 12 hr. Cuttings were treated with Dip-N-Grow rooting solution (1% indole-3-butyric acid, 0.5% 1-Naphthaleneacetic acid) (Griffin Greenhouse Supplies, Tewksbury, MA, USA), and held in a mist bed (Caswell et al. 2008; Ingwell and Preisser 2011; McKenzie et al. 2014). After six months in the mist bed, rooted plants were potted in 4 liter pots with the same soil mix as controls and placed outdoors between greenhouses under shade cloth next to control plants.

In April 2013, 64 of the potted HWA-resistant saplings and 32 of the susceptible hemlocks were transplanted into an outdoor raised bed 30 m long by 1 m wide and 0.5 m deep in partial shade on the north side of the URI campus greenhouse. The bed was filled with a 1:1 mix of soil and compost and supplemented with soil from a nearby hemlock stand. All saplings were grown in this bed until May 2015 when they were dug and all soil was washed from roots before potting into 15 liter nursery pots with a growing media of composted peanut hulls. The potted plants were then grown under 75% shade between greenhouses through the summer of 2015.

Reforestation plot establishment

In September 2015, all trees used in the field trial were dug, balled, and burlapped. Trees were then treated with dinotefuran (Safari 20 SG) as a bark spray at ½ the lowest label rate (0.65 ml/1 L), following a series of soap and oil treatments, to

ensure that trees were free of living HWA and elongate hemlock scale (*Fiorinia externa*; EHS) prior to transport and planting. The efficacy of Safari 20 SG has been shown to decline to zero within two years of treatment (Joseph et al. 2011), meaning that the treated trees would have been vulnerable to both pests within 1-2 years of planting.

Eight HWA-resistant trees (four propagules from each of parent trees #3 and #4) and four HWA-susceptible trees (two from PA-collected seeds and two from MI-collected seeds) were planted in each of eight field plots located throughout the range of HWA in the northeastern USA (Fig. 1). Size- and age-matched saplings were provided to cooperators in 15-liter pots for planting at the following locations ordered by latitude in Fig. 1: Cornell University, Ithaca, NY (site 1), Arnold Arboretum, Boston, MA (site 2), Yale-Myers Forest, Hamden, CT (site 3), Tiadaghton State Forest, Lycoming County, PA (site 4), New York Botanical Garden, Bronx, NY (site 5), Cunningham Falls State Park, Thurmont, MD (site 6), Kanawha State Forest, Charleston, WV (site 7), and the Mountain Research Station, Waynesville, NC (site 8). All plots were established either within or adjacent to forests containing HWA-infested hemlocks. Test saplings were planted randomly in the plots approximately three meters apart. Where needed, plots were enclosed in a deer fence. Following planting, plots were left undisturbed for four years.

Plot surveys

In fall 2019, we returned to each plot and evaluated the saplings for survival and growth. Sapling growth metrics included tree height (total height of stem from ground level to apical growth tip), lateral growth (length of one lateral branch

emerging from each of five side branches), drip line (length of longest non-apical terminal branch), DBH (trunk diameter at 5 cm aboveground), and condition (% foliage remaining). Condition was quantified via a 5-to-0 scale: 5 = zero to 20% foliage loss, 4 = 21-40% loss, 3 = 41-60% loss, 2 = 61-80%, 1 = 81-99% loss, 0 = dead. To assess HWA and EHS densities, five lateral branches emerging from each of five side branches at varying heights were haphazardly selected on each sapling, and the total number of HWA sistens and EHS on 5 cm terminal growth were counted.

Statistical analysis

R software v. 3.5.0 was used for all statistical analyses (R Development Core Team, 2018). Site-level mean survival rate and condition were evaluated among all live and dead trees, using linear mixed models in *lme4* (Bates et al. 2014). Each model term was tested for significance using Type II Wald chi square tests in *car* (Fox and Weisberg 2011). Growth metrics, HWA densities, and EHS densities were evaluated in a second analysis including only live saplings, using linear mixed models in *lme4*. Significance tests were done using Type II Wald chisquare tests in *car*. Graphics were created in *ggplot2* (Wickham 2016).

Results

Ninety-six percent of HWA-resistant hemlocks survived, compared to 48% of the control plants ($t = 3.07$, $X^2 = 9.44$, $p = 0.02$). A comparison of surviving trees found that the HWA-resistant plants were 32% taller ($t = 3.25$, $X^2 = 10.5$, $p < 0.01$; Fig. 2A), put out 18% more lateral growth ($t = 2.40$, $X^2 = 5.80$, $p = 0.01$; Fig. 2B), had 20% longer drip lines ($t = 2.26$, $X^2 = 5.13$, $p = 0.02$; Fig. 2C), and were in 58% better condition ($t = 3.87$, $X^2 = 14.9$, $p < 0.01$; Fig. 2D) than the control plants. There were,

however, no significant between-treatment differences in stem diameter ($t = 0.38$, $X^2 = 0.14$, $p > 0.05$).

Trees in all eight plots were found to be infested with EHS. The HWA-resistant hemlocks had EHS densities 60% lower than those of the controls ($t = -2.53$, $X^2 = 6.44$, $p < 0.01$; Fig. 2E). HWA was only found on trees at three out of the eight reforestation plots. The density of HWA on HWA-resistant eastern hemlocks was 35% lower than on HWA-susceptible hemlocks ($t = -0.30$, $X^2 = 0.09$, $p > 0.05$; Fig. 2F); the lack of statistical significance likely reflects the small sample size.

Discussion

We found that HWA-resistant eastern hemlocks outperform HWA-susceptible eastern hemlocks when trees of both types are planted in HWA-infested forests, a result that may prove critical in HWA management. We found that HWA-resistant eastern hemlocks had significantly higher survival, better growth (Fig. 2A – C), and retained more foliage (Fig. 2D) than HWA-susceptible hemlocks. Biological control and insecticide treatment, the two primary approaches to HWA management, have not been completely effective at mitigating the impact of HWA on eastern hemlock forests (Limbu et al. 2018; Sumpter et al. 2018); as a result, HWA-induced hemlock mortality has severely affected ecosystem dynamics in eastern U.S. forests. Eastern hemlock supports critical habitat for unique vertebrate and invertebrate communities (Ellison et al. 2010); dramatic shifts in hydrological regimes, soil nutrient cycling, and understory vegetation, all caused by hemlock decline, may have long-lasting changes that compromise these areas (Orwig et al. 2008). The fact that HWA-resistant eastern hemlocks grown in HWA-infested forest plots did better than HWA-susceptible trees

suggest that HWA-resistant hemlocks should be considered for inclusion in future reforestation efforts aimed at returning this tree to an ecologically significant component of eastern U.S. forests.

While the difference in survival and condition was striking, the observed variation in pest densities is equally important. HWA-resistant eastern hemlocks supported 60% lower EHS densities (Fig. 2E) and 35% lower HWA densities (Fig. 2F) than HWA-susceptible trees. These differences may result from the higher terpene concentrations in the twigs and needles of the HWA-resistant trees, clonal propagules of the same trees tested in McKenzie et al. (2014). Plants respond to stylet-feeding insects via an array of metabolic changes (reviewed in Howe and Jander 2008), and terpene accumulation is a primary chemical defense of conifers against herbivory (reviewed in Mumm and Hilker 2006). There is strong circumstantial evidence that terpenes affect HWA resistance: the terpene profiles of HWA-resistant *Tsuga* species differ substantially from those of HWA-susceptible species (Lagalante and Montgomery 2003). Increased terpene concentrations in both eastern hemlock and the Japanese species *T. sieboldii* are also linked to decreased EHS fecundity (McClure and Hare 1984). Regardless of mechanism, lower densities of sap-feeding herbivores may also indirectly provide protection against other consumers. Results of field and laboratory research indicate that gypsy moth (*Lymantria dispar*) and hemlock looper (*Lambdina fiscellaria*) preferentially consume, and have higher fitness on, HWA-infested eastern hemlocks (Kinahan et al. 2020; Wilson et al. 2016). Although we did not assess folivore densities, outplantings of HWA-resistant hemlocks may thus also be more likely to survive outbreaks of other pest species.

As with any experiment, there are several caveats that should be mentioned. First, while we made sure that our HWA-resistant and HWA-susceptible hemlock saplings were healthy and grown under similar conditions, and attempted to match all experimental plants in terms of size and age, we did not record pre-experiment data on plant height or other variables. Without this data, we cannot be certain that variation in initial plant height or dripline did not contribute to current treatment-level differences in these variables. Were this the case, however, we would also have expected to find significant differences in trunk diameter; the fact that HWA-resistant and HWA-susceptible trees did not differ in this variable suggests that any initial between-treatment variation, if present, was minimal.

Second, the absence of monitoring between the 2015 start of the experiment and our 2019 resurvey means that we cannot definitively link increased mortality of HWA-susceptible trees to higher pest densities. While we lacked the funding necessary to conduct such surveys, the lower pest densities on, and higher survival of, HWA-resistant hemlocks are nonetheless consistent with herbivore-driven tree mortality. Finally, the relatively low number of trees planted at each site (eight HWA-resistant and four HWA-susceptible saplings) prevented us from conducting detailed site-level analyses. This limitation means that our work is most appropriately viewed as a ‘proof of concept’ experiment highlighting the need for future research exploring how HWA-resistant eastern hemlocks might best be integrated into existing HWA management efforts.

Prior to the accidental introduction of HWA, eastern hemlock was one of the most abundant, long-lived, and ecologically significant trees in the eastern U.S.

(Ellison et al. 2005). Hemlock-dominated forests were characterized by deep shade, acidic, slowly-decomposing soil, and a cool microclimate, which together created unique and critical habitat for many terrestrial and aquatic species (Ellison et al. 2005; Orwig et al. 2008). Unfortunately, neither biological control and insecticide treatments have effectively protected hemlock from HWA (Limbu et al. 2018; Sumpter et al. 2018) or prevented the spread of this pest: HWA was recently detected for the first time in western Michigan, and has spread north into Nova Scotia. This has resulted in the widespread loss of hemlock, with more than a 60% decrease in total hemlock basal area since 1997 in New England alone (Gómez et al. 2015). This loss, in combination with the virtual absence of hemlock regeneration in HWA-infested areas (Preisser et al. 2011), has drastically changed native forest communities. Hemlock-associated forests are now characterized, for instance, by novel understory vegetation communities (Ingwell et al. 2012), and significantly reduced soil moisture and C:N ratios (Orwig et al. 2008). Our results suggest that HWA-resistant eastern hemlocks may, by their higher survival and lower pest densities, play a role in restoring hemlock-associated forest dynamics. These findings, in combination with previous work on the HWA-resistant trees we used, argue strongly for additional research exploring the identification, characterization, and use of HWA-resistant eastern hemlocks in current and future reforestation efforts.

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Figure Legends

Figure 1. Locations of the eight field plots (numbered by latitude) containing HWA-resistant and HWA-susceptible eastern hemlock saplings planted in HWA-infested forests.

Figure 2. Mean \pm 1 SE height (A), lateral growth (B), drip line (C), condition (D), EHS densities (E) and HWA densities (F) of HWA-resistant and HWA-susceptible eastern hemlocks grown in HWA-infested field plots for four years.

Figure 1.

Sites

1. Cornell University,
Ithaca, NY
(42.6106, -76.4397)
2. Arnold Arboretum,
Boston, MA
(42.2998, -71.1243)
3. Yale-Myers Forest,
Hamden, CT
(41.9589, -72.1612)
4. Tiadaghton State
Forest, Lycoming
County, PA
(41.3190, -77.3867)
5. New York Botanical
Garden, Bronx, NY
(40.8623, -73.8772)
6. Cunningham Falls
State Park, Thurmont,
MD
(39.6009, -77.4503)
7. Kanawha State Forest,
Charleston, WV
(38.3387, -81.6041)
8. Mountain Research
Station, Waynesville,
NC
(35.4871, -82.9680)

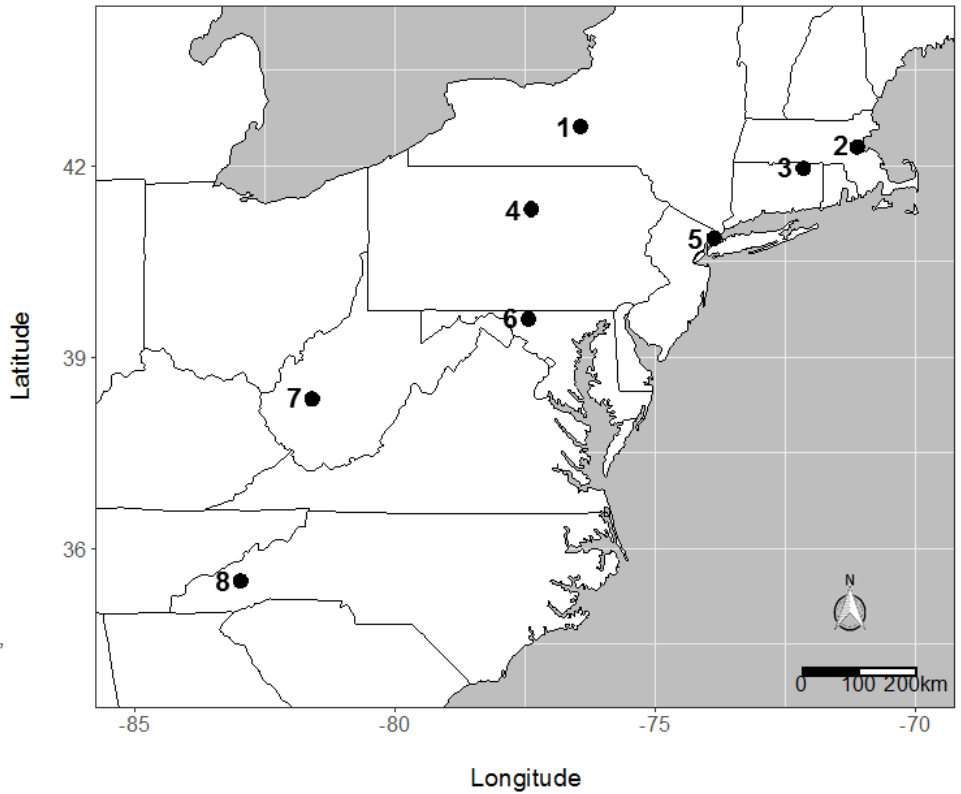


Figure 2.

